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RESEARCH PAPER

Effect of corn herbicide on alfalfa planting in the black soil area of Northeast China

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Abstract

Corn and alfalfa intercropping is considered an important practice for controlling soil erosion. The effects of herbicide application during the emergence and growth periods of alfalfa have not yet been studied. Therefore, this study was conducted to determine the impacts of two herbicides (nicosulfuron and atrazine) on alfalfa at different growth stages (before emergence, at emergence, and during the branching stages). The results revealed that herbicide application to maize significantly decreased emergence, plant height, stem diameter, biomass and grain yield. The mortality rate of alfalfa during the first year's branching stage was the lowest, indicating that tolerance to this herbicide improved. The herbicides had negative impacts in a dose-dependent manner; for example, the same dose effects gradually increased first but then decreased. Moreover, the root-to-seedling ratio, activity index, and contents of soluble sugars and soluble protein in alfalfa significantly decreased in all three treatment groups, whereas the activities of three antioxidant enzymes significantly increased. The residual effect of the herbicide in the field completely disappeared when the interval between applications exceeded eleven months. Therefore, these findings indicate that herbicide application has negative effects on alfalfa growth.

Keywords

Antioxidants, alfalfa, growth, herbicide, maize, seedlings, yield

Introduction

The increase in population has led to increased use of natural resources and unreasonable cultivation, which is degrading the black soil in Northeast China (Liu 2018). The special landform in the black soil area in Northeast China is prone to water erosion during the rainy season, resulting in erosion channels and the encroachment of cultivated land. Moreover, gale-force winds in spring erode the exposed surface, peel the topsoil, deteriorate the soil structure and reduce soil fertility, resulting in serious food production problems (Liu et al. 2010). In China, corn, as the primary food crop, has the largest planting area, and herbicides, owing to their high efficiency and ability to be used over large areas, have become the preferred choice for weed control in corn fields (Janak and Grichar 2016; Liu et al. 2020). The most widely used sulfonylureas are nicosulfuron single agents and nicosulfuron and atrazine mixed agents (Li et al. 2023). The main mechanism of weed control is the inhibition of acetolactate synthase (ALS), which can effectively prevent the synthesis of three branched chain amino acids (valine, leucine and isoleucine), prevent weed cell division,

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and stop the growth of weeds (Williams et al. 2014; Li Yang et al. 2018; Leite et al. 2020; Zhu et al. 2023). Atrazine inhibits photosynthesis and electron transfer and reduces the photosynthetic efficiency of weeds, thus effectively controlling them (Gaston et al. 2003; Cheron et al. 2022).

A recent study revealed that atrazine has good control effects on weeds in corn fields and can significantly increase plant yield (Liu et al. 2024). The application of nicosulfuron and other herbicides alone and the mixing of nicosulfuron and other herbicides with atrazine promoted the absorption of magnesium and other elements in plants but also increased the content of polysaccharides such as maltose and sucrose while increasing the content of monosaccharides. This in turn affects the nutritional quality of crops (Cutulle et al. 2018). Mesarovic et al. (2019) applied different herbicides, such as nicosulfuron, to three different hybrid maize varieties. They reported that nicosulfuron alone could change the nutritional status of maize grains (Mesarović et al. 2019). Liu et al. (2019) reported that the application of brassinosteroid insecticides reduced the degree of injury caused by nicosulfuron to crops, improved the yield and photosynthesis of maize, and significantly improved the tolerance of maize to nicosulfuron (Liu et al. 2019). Intercropping of maize and legume crops can increase the leaf area index, improve the water utilization rate in soil (Ogindo and Walker 2005), increase the accumulation of organic matter in soil, and improve soil fertility (Fustec et al. 2010). Intercropping also improves the utilization of nitrogen, phosphorus and potassium (Zhang and Wangyu 2014). The intercropping mode of potato (*Solanum tuberosum* L.) and oat (*Avena sativa* L.) promoted the activities of soil microorganisms and reduced the residue of mineral nitrogen in the soil (Yu 2023). Studies at home and abroad have shown that the use of the corn-alfalfa intercropping mode in sloped farmland can effectively trap sediment and reduce slope runoff, thus reducing soil and water loss (Alegre and Rao 1996; Wang et al. 2022).

In recent years, there have been few reports on the effects of herbicides on alfalfa, especially on the screening of herbicides in corn-alfalfa intercropping. After 18 kinds of herbicides were applied to alfalfa, Duan et al. reported that herbicides such as ethoxyflufen cream, fluosulfanilamide water agent and oxazone cream were protoporphyrinogen oxidase inhibitors and were not safe for alfalfa (Duan et al. 2015). An earlier study also revealed that the photosynthetic rate and stomatal conductance of alfalfa decreased after the application of atrazine and that inoculation with *Funneliformis mosseae* effectively alleviated the harm caused by herbicides (Fan et al. 2018). The efficiency of herbicides in controlling weeds has been well reported; nevertheless, their nontargeted toxicity can damage soil health by disrupting soil microbial communities. Therefore, to mitigate these challenges, herbicides must be integrated with different amendments. For example, coupling herbicides with soil amendments, including biochar, can sequester herbicides and reduce their leaching and persistence, thereby accelerating herbicide degradation and rehabilitating the soil (Xie et al. 2024; Jena et al. 2025). Researchers have also reported that biochar and nanomodified biochar increase the anatomical features of roots and leaf tissues and help improve soil health and mitigate the toxic impacts of abiotic stresses (Abbas et al. 2024; Gulsah et al. 2025; Sultan et al. 2025). Alfalfa, as a high-quality forage, not only can solve the grain-grass conflict in the northern agro-pastoral zone in corn-alfalfa intercropping but can also play a positive role in soil and water conservation. The aim of this study was to provide strong evidence for the application of herbicides in the control of weeds in corn croplands via corn-alfalfa intercropping. This study also provides a scientific reference for the rational planting and application of alfalfa in soil and water conservation and sustainable agricultural development.

Materials and methods

Experimental site

This research was conducted at the Songnen Grassland Ecological Research Station of Northeast China Normal University, which is located in the southern part of the Songnen Plain and the territory of Waijingzi Stud Farm in northwestern Changling County, Songyuan city, Jilin Province, at an altitude of approximately 140 m. Its geographical coordinates are approximately 44°45'N, 123°45'E, and it has a semihumid and semiarid temperate monsoon climate. The annual average temperature is 4.6~6.4 °C, the annual accumulated temperature is 3000~3500 °C, and the average daily temperature is higher than 10 °C for approximately 130 days throughout the year. The frost-free period is approximately 150 days. The annual precipitation at this test site is approximately 400–500 mm, and the precipitation is concentrated mainly from June to August every year. The soil types mainly include chernozem soil, black soil, meadow soil, aeolian sand soil, saline soil, alkaline soil, etc. The soil at this test site is aeolian sand soil, which is barren and has a low organic matter content and poor water (Table 1).

Table 1. Soil basic nutrients in the test area.

	Electric conductivity	Organic matter	Total nitrogen	available potassium	available phosphorus
pH	us/cm	g/kg	g/kg	mg/kg	mg/kg
7.76	88.29	8.21	0.86	187.63	15.2

Test variety

In 2019, the new grass variety 'Dongmu No.1' was approved and regisetered by the National Grass Variety Committee as a new grass variety in Chian, with Northeast Normal University as the breeder. The plant type is upright, compact, has a high grass yield, has strong stress resistance (drought resistance, cold resistance, salt and alkali resistance), and has a 1000-grain weight of 2.24 g.

Pilot herbicides

In this study, nicosulfuron·atrazine, a special herbicide produced by Shandong Aokun Crop Science Co., Ltd., was selected. Its effective ingredients were 25% atrazine and 5% nicosulfuron.

Experimental design and methods

Indoor seed germination experiment

The seed germination experiment was conducted at the Seed Ecology Laboratory of Northeast Normal University via the laboratory paper germination method (TP) (MacArthur Clark and Sun 2020). Alfalfa seeds of uniform size and full grain size were selected, immersed and disinfected with 0.1% mercury for 5 min, and then rinsed repeatedly with sterile water until the pH test paper was neutral (Zhang et al. 2021). Petri dishes with a diameter of 90 mm were selected for this experiment, and two layers of filter paper with a diameter of 90 mm were laid on the bottom. Fifty seeds were evenly distributed in each Petri dish. Three gradients were set: (high) dose (100.00%, the recommended concentration of herbicide was 1200 ml/hm^2), medium dose (66.67% of the normal dose) and low dose (33.33% of the normal dose). Another group was set up with water as the control (CK), which was repeated 4 times. All Petri dishes were placed in an intelligent incubator with 12 h light and 12 h darkness. The amount of water lost was measured every day and supplemented over time. The seed germination test was completed on the 10th day (Wu et al. 2011; Niste et al. 2015; MacArthur Clark and Sun 2020).

Field experimental design

A corn field was selected from the Northeast Normal Grassland Experimental Station. The planting times of corn and herbicide are fixed annually, whereas alfalfa can be sown at various times because of its short growth cycle. Therefore, determining the alfalfa growth stage during corn herbicide application is challenging. Therefore, three different periods were set for treatment in the first year of the experiment. Before emergence (June 25, 2018), emergence (July 15, 2018) and branching (August 10, 2018) of alfalfa after seeding. Moreover, in corn-alfalfa intercropping plots, corn herbicide can be sprayed directly onto cultivated land, or different doses of corn herbicide can be sprayed onto cultivated land adjacent to alfalfa in a medium variety of situations. Therefore, three doses of herbicide are used for each period, which are normal (high) doses (the recommended concentration of herbicide is selected), and the recommended concentration of herbicide is selected. 1200 ml/hm^2), a medium dose (2/3 of the normal dose) and a low dose (1/3 of the normal dose). Each treatment consisted of 9 treatments for 3 different periods × different doses. Another group was sprayed with the same amount of water as the control (CK), for a total of 10 treatments, and each treatment was repeated 3 times.

There were a total of 9 experimental cells, each cell was 2 × 4 m, a completely random block design was adopted between each treatment, and a 30 cm isolation strip was set up between each cell. On June 25, 2018, the seeds were sown at a row spacing of 30 cm, and the sowing amount was 15 kg/hm^2. Corn herbicide was applied in the morning or evening on sunny, windless days. When herbicide is applied, plastic sheets were used to separate adjacent plots to prevent the herbicide from blowing in the wind. The experiment was performed in a randomized complete block design with a factorial arrangement with three replications.

Field experiment in the following year

In May of the following year, the treatment plots were kept unchanged, a small part of the returned alfalfa was dug out with roots (most of the alfalfa was not returned to green), and the ditch was reopened on the right side of the original alfalfa row 15 cm, with the same sowing distance and sowing amount as in 2018, to explore the residual effect of this corn herbicide.

Measurement items and methods

Laboratory test

Seed germination indexes

From the beginning of seeding, the number of seeds that germinated in each Petri dish was counted every 24 hours, and the germination rate, germination potential and germination index were calculated.

$$\text{germination rate}(\%) = \frac{\text{Number of germinated seeds}}{\text{Number of test seeds}} \times 100\%$$

$$\text{Germination potential}(\%) = \frac{\text{Germination peak seed number}}{\text{Number of test seeds}} \times 100\%$$

(Bhattarai et al. 2020)

where Germination potential = $\sum \frac{G_t}{T_t}$, G_t represents the number of seeds that germinated in t days, and T_t represents the number of days after implantation (Maguire 1962).

On day 10 of the experiment, 10 alfalfa seedlings with uniform growth were randomly selected from each Petri dish, their root length and seedling length were measured with Vernier calipers, and the root-shoot ratio and vitality index were calculated.

$$\text{Root-shoot ratio} = \frac{\text{Root length(mm)}}{\text{Seedling length(mm)}}$$

$$\text{Vitality index} = \text{Germination index} \times (\text{root length} + \text{seedling length})$$

(Bhattarai et al. 2020).

Field test

Relative emergence rate

After treatment on the day of seeding, the emergence of alfalfa was investigated from the 3rd day, the emergence time of alfalfa was recorded, and the relative emergence rate was calculated from the number of seedlings on the 21st day.

$$\text{Relative emergence rate}(\%) = \frac{\text{Number of seedling emergence in treated plots}}{\text{Number of seedling emergence in control plots}} \times 100\%$$

Status of main agronomy

On the 21st, 28th, 47th, 55th, 62nd and 77th days (initial flowering stage) after seeding, 10 uniformly growing alfalfa plants (30 in total for 3 repetitions) were randomly selected from each plot, and their height was measured with a tape.

The stem thickness under the first branch of the main stem of 10 alfalfa plants was measured with Vernier calipers. The number of basal branches on 10 alfalfa plants was investigated.

Victimization

On the 21st, 28th, 47th, 55th, 62nd and 77th days after seeding, alfalfa with uniform growth of 1 meter in length was selected in each plot, and its damage situation was divided into 5 levels as follows:

Grade I: good plant growth, dark green leaves
Grade II: Growth is inhibited and can continue to grow
Grade III: Leaf atrophy, yellow leaf color, growth deformity
Grade IV: Severely inhibited, unable to resume growth
Grade V: Complete death of the plant

The proportion of each level of damage was calculated.

Number of plants

On the 77th day after seeding, 1 m2 of alfalfa (row spacing of 0.3 m, converted to 3.33 m in length) with uniform growth was selected from each plot to investigate the number of plants.

Production

On the 77th day after sowing, uniformly growing alfalfa plants within a 1 m2 quadrat were sampled from each test plot, sealed in selfsealing bags, and immediately weighed to determine the fresh weight.

Each cell consisted of 200 g of alfalfa, which was placed in a 105 °C oven and dried in a 65 °C oven for 48 h, after which the weight of each area was removed, and the moisture content of each area was calculated.

$$\text{Water content}(\%) = \frac{(\text{Fresh weight-Biomass})\text{g}}{\text{Fresh weight}(\text{g})} \times 100\%$$

The amount of water in each neighborhood is calculated, and the amount of biomass (production) in each neighborhood is calculated.

The production of the test was the first year after the alfalfa was planted.

Single strain and strain

Through the fresh weight of each area, the number of plants, the number of plants, the weight and the strain of the alfalfa single strain were calculated.

$$\text{Fresh weight per plant}(\text{g}) = \frac{\text{Fresh weight above ground}(\text{g})}{\text{The number of plants}}$$

$$\text{Biomass per plant}(\text{g}) = \frac{\text{Above-ground biomass}(\text{g})}{\text{The number of plants}}$$

Seedling expectations

For the 10-d experiment, 10 long homogeneous alfalfa seedlings were randomly selected from each culture dish, and the content of soluble sugars in the seedling tissue was determined via the anthranone color method.

The content of soluble protein in the seedling tissue was determined via Coomassie blue staining.

The proline content in the seedling tissue was determined via acid ninhydrin staining.

The content of malondialdehyde (MDA) in the seedling tissue was determined via the thiobarbituric acid method.

The activity of superoxide dismutase (SOD) in the seedling tissue was determined by using nitroblue tetrazolium (NBT).

The activity of peroxidase (POD) was detected via another technique.

The active concentration of catalase (CAT) in the seedling tissue was determined via ultraviolet spectrophotometry.

Data processing and analysis

All the data were analyzed via two-way analysis of variance (ANOVA) via SPSS 26.0 (version 26.0 SPSS Inc., Chicago il, sa) software. Differences among means were determined via the Duncan test at a significance level of $p = 0.05$.

Results

Effects of different doses on the alfalfa germination rate and related parameters

There was no significant difference between the low-dose herbicides and the control ($p > 0.05$). The high-dose treatment decreased the germination of alfalfa seeds by 17.86%, 16.57% and 18.44%, respectively. Moreover, after 9 days of low- and high-dose treatment, alfalfa root growth increased by 25.61%, 47.15% and 52.85%, respectively (Fig. 1).

Effects of different dosages of herbicides on four kinds of organic matter in alfalfa

Compared with those in the control, there were significant changes in the amounts of proline, propylene, soluble sugar and soluble protein in the plants treated with low-, medium- and high-dose herbicides. The low-, neutral- and high-dose treatments decreased the proline content by 36.58%, 52.97% and 60.27%, respectively. In the control group, the content of alfalfa was 17.87 mol/g3, and the herbicide dose decreased the proline content to 26.30%, 17.24% and 8.06%, respectively. The soluble sugar content of the control group

was 0.53 mg/g3, and the three treatments decreased the sugar content by 81.13%, 81.26% and 80.60%, respectively. The content of soluble protein in the control group was 16.35 mg/g3, and the soluble protein content of the three types of alfalfa decreased by 75.66%, 63.73% and 51.99% with different rates of herbicide application (Fig. 2).

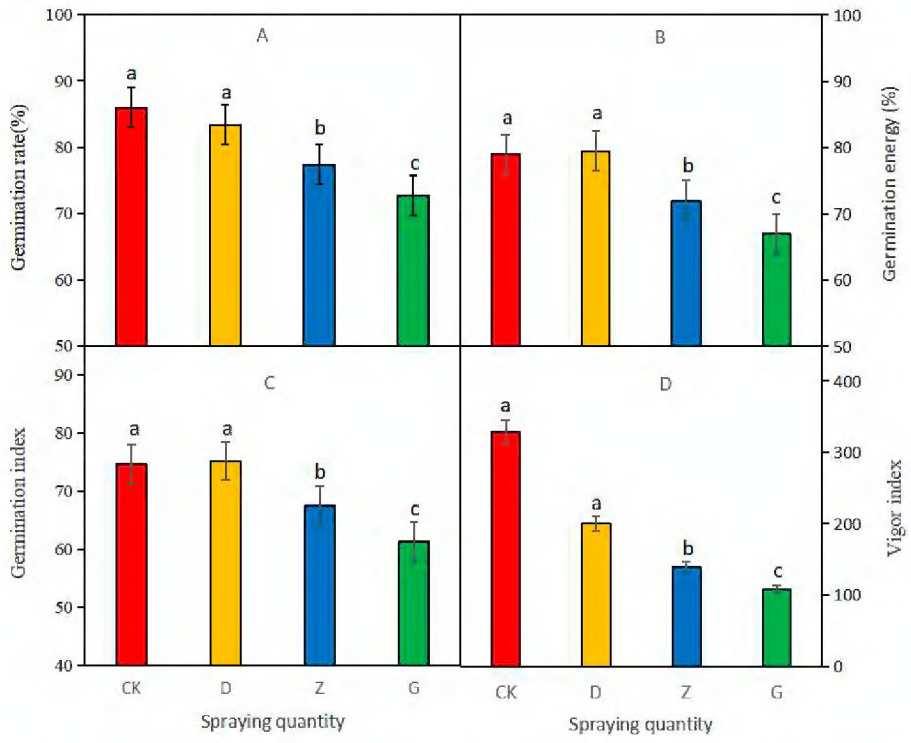

Figure 1. Effects of different doses on the germination of alfalfa seeds. Note: The values indicate means (n = 3) with ± SE, and the same letters indicate non-significant differences at $p > 0.05$. Different letters indicate significant differences at $p < 0.05$.

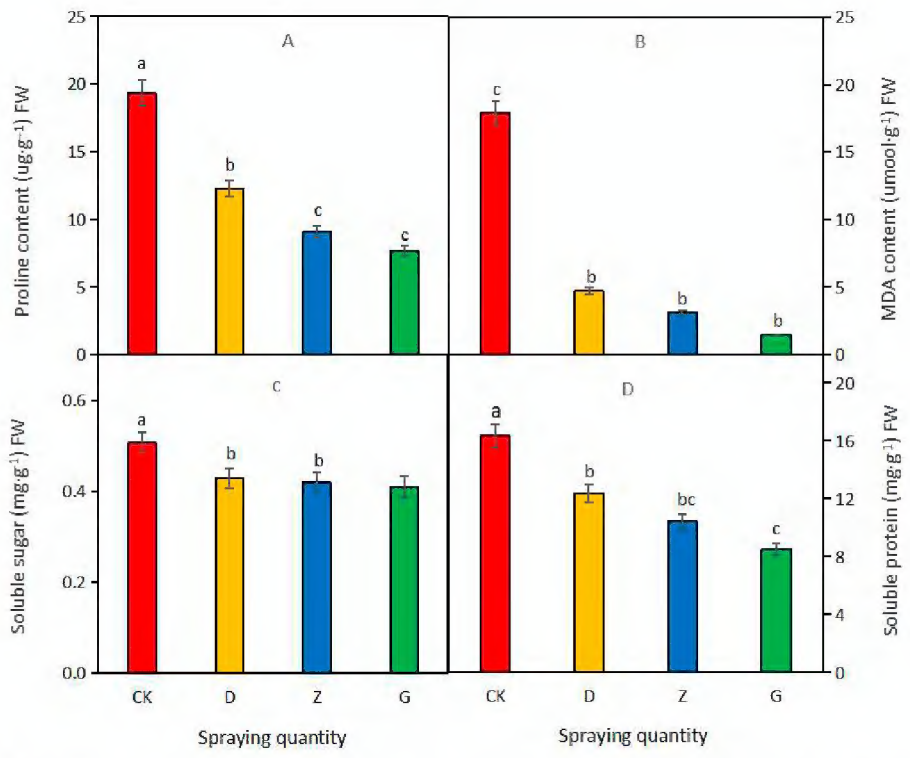

Figure 2. Effects of different spraying doses on the four organic matter contents of alfalfa. Note: Note: The values indicate means (n = 3) with ± SE, and the same letters indicate non-significant differences at $p > 0.05$. Different letters indicate significant differences at $p < 0.05$.

The effects of different dosages of herbicides on the activity of the 3 antioxidant enzymes in alfalfa

Compared with the control, the different herbicide treatments increased the antioxidant activity. Among them, the peroxides in the control group were 508.94 U/g, and low, neutral and high doses increased POD activity by 128.88%, 134.98% and 133.89%, respectively. The three different dosage treatments increased the SOD activity by 140.03%, 146.41% and 142.66%, respectively. Moreover, the three herbicides increased the CAT activity by 141.34%, 142.86% and 154.72%, respectively, compared with that of the control (Fig. 3).

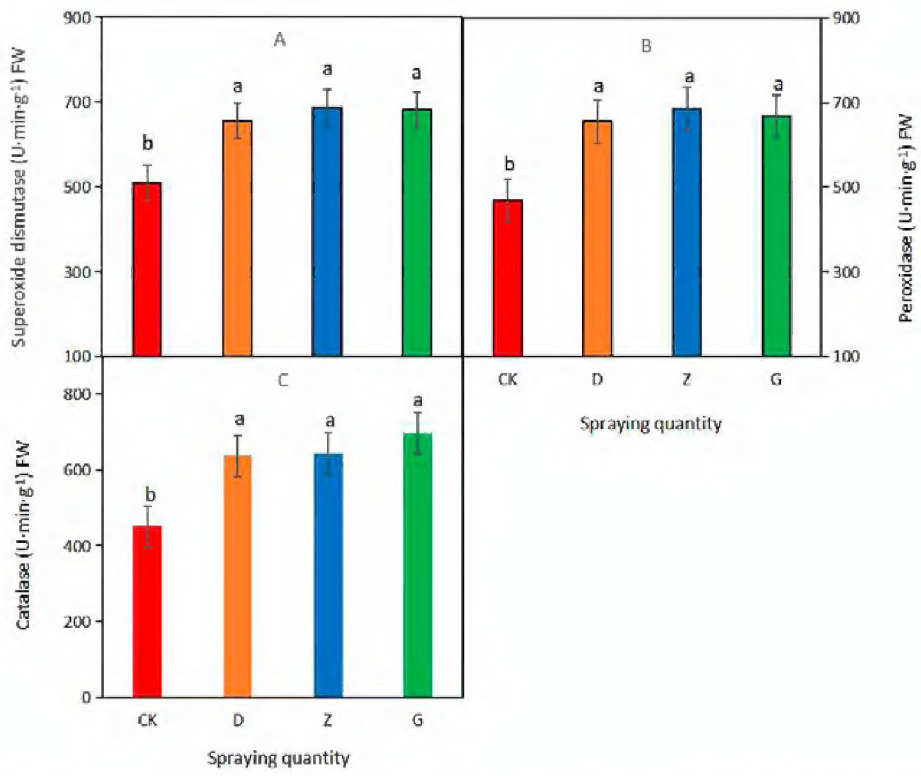

Figure 3. Effects of different spraying doses on the three antioxidant catalase genes in alfalfa. Note: The values indicate means (n = 3) with ± SE, and the same letters indicate non-significant differences at $p > 0.05$. Different letters indicate significant differences at $p < 0.05$.

Effects of different doses of herbicides before sowing on the emergence and aboveground vegetative growth of alfalfa

This study revealed that spraying with three different doses of herbicide affected the emergence time of alfalfa. As of July 2 (8d), more than 50% of the alfalfa seedlings in the control community had emerged, while none of the seeds had emerged at the three different doses. As of July 10 (16d), the emergence rate of alfalfa in the control area was approximately 85%, while the seedlings were occasionally produced at three different doses after sowing, and the emergence rate was very low.

The effects of different dosages of herbicides on the relative seedling rate of alfalfa before sowing

The study revealed that the seedling rate of alfalfa was significantly affected by the herbicide dose and decreased as the herbicide dose increased. The relative seedling rate of the herbicide treatment area was 13.12%, and the relative seedling rate of the herbicide treatment area was 13.12%. The relative seedling rates of the herbicide at the injection dose (66.67%) and high dose (100.00%) were only 4.87% and 2.93%, respectively (Fig. 4).

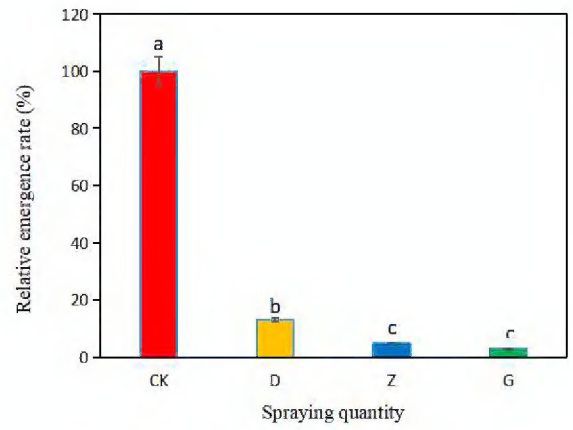

Figure 4. Effects of different spraying doses at preemergence on the seedling emergence rate of alfalfa. Note: The values indicate means (n = 3) with ± SE, and the same letters indicate non-significant differences at $p > 0.05$. Different letters indicate significant differences at $p < 0.05$.

Effects of different dosages of herbicides on purple flowers of high-density alfalfa strains before and after production

The results showed that spraying this herbicide before emergence had a significant effect on the plant height of alfalfa ($p < 0.05$). The height of the alfalfa plants in the control plot was significantly greater than that in the control plot, and the medium- and high-dose treatments decreased the plant height by 31.08%, 25.88% and 15.89%, respectively (Fig. 5A).

The results showed that spraying three different doses of herbicides before the first year of emergence had no harmful effect on alfalfa sown in the next year. On the 75th day after sowing in the following year, the height of the alfalfa plants in the control plot was 52.80 cm, and the herbicide decreased the height of the plants by 102.86%, 103.45% and 103.03%, respectively (Fig. 5B).

Effects of spraying different doses of herbicides before emergence on the stem diameter of alfalfa

The stem diameter of the alfalfa plants in the control plot was 1.03 mm, and after the 55th day, the difference in stem size between the control plot and the three different treatment plots gradually decreased. By day 77, there was no significant difference in stem size between the three treated plots and the control plots (Fig. 5C, D). The results revealed that after three different herbicide doses were sprayed before seedling emergence in the first year, the stem diameter of alfalfa in the next year was significantly greater than that of the control ($P < 0.05$).

Effects of spraying different doses of herbicides before emergence on the number of basal branches of alfalfa

After herbicide spraying before emergence, there was no significant difference in the number of basal branches of alfalfa between the control plot and the three treatment plots (Fig. 5E). The results revealed that spraying three different doses of herbicides before the first year of emergence had no significant effect on the number of basal branches sown in the next year (Fig. 5F).

Effects of spraying different doses of herbicides before emergence on alfalfa yield and other indicators

The number of alfalfa plants in the control plot was 2,696,800 plants/hm^2, and the number of plants in the low-dose, medium-dose and high-dose plots decreased by 13.73%, 5.11%

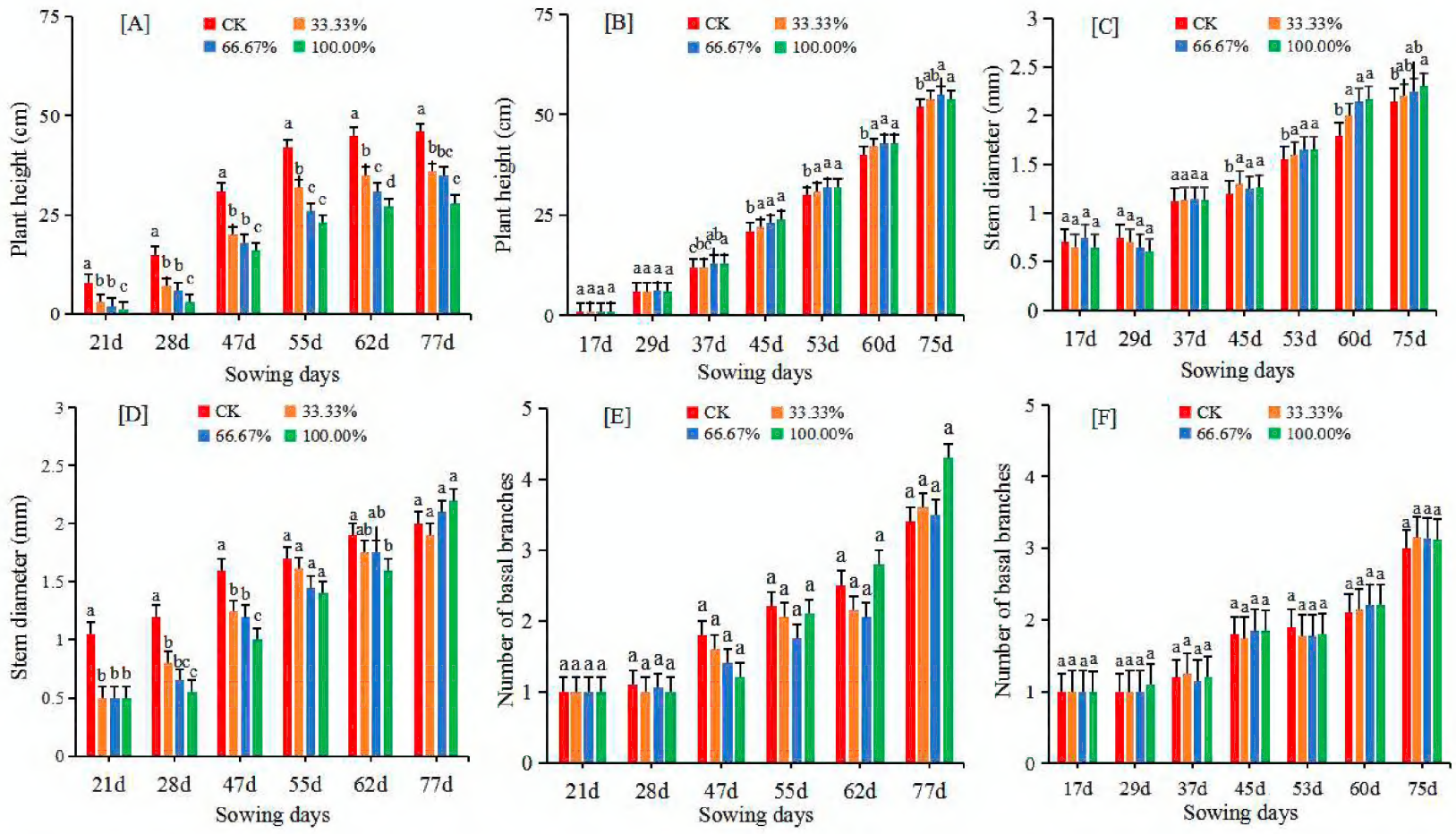

Figure 5. Effects of different spraying doses at preemergence on plant height, stem diameter and number of basal branches of alfalfa. Note: The values indicate means (n = 3) with ± SE, and the same letters indicate nonsignificant differences at $p > 0.05$. Different letters indicate significant differences at $p < 0.05$.

and 3.30%, respectively. . The aboveground fresh weight of the alfalfa in the control plot was 6227.99 kg/hm^2. The aboveground fresh weight in the low-dose and high-dose plots decreased by 35.04%, 11.72% and 7.27%, respectively, of that in the control plot. The aboveground dry weight of the alfalfa in the control plot was 1717.59 kg/hm2. The above-ground dry weights of the plots treated with the low, medium and normal doses decreased by 36.16%, 11.81% and 7.58%, respectively. In addition, the fresh weight per plant in the control plot was 2.31 g, and the fresh weight per plant in the low-dose treatment, medium-dose treatment and normal-dose treatment groups decreased by 256.28%, 229.00% and 221.21%, respectively. The dry weight per plant of alfalfa in the control plot was 0.63 g, which was decreased by 266.67%, 233.33% and 233.33%, respectively. In the following year, the aboveground fresh weight of alfalfa in the control plot was 7101.49 kg/hm2, and the aboveground fresh weights of the three treatments increased by 28.83%, 25.64% and 32.34%, respectively. The aboveground dry weight of alfalfa in the control plot was 2058.99 kg/hm^2, and the aboveground dry weights of the three treatments increased by 29.54%, 29.03% and 27.84%, respectively. The fresh weight per plant of alfalfa in the control plot was 3.47 g, and the fresh weight per plant in the three treatments increased by 22.57%, 18.16% and 24.14%, respectively. The dry weight per plant of alfalfa in the control plot was 1.01 g, and the dry weight per plant in the three treatments increased by 23.08%, 21.34% and 19.60%, respectively, compared with those in the control plot.

Effects of low-dose treatment on alfalfa

The results revealed that alfalfa experienced the greatest loss on the 21st day after a low dose of herbicide was sprayed before emergence. The results revealed that 88.27% of the alfalfa plants died completely, 5.33% of the alfalfa plants grew normally, and 6.40% of the alfalfa plants were inhibited but could continue to grow. With the passage of time, the herbicide effect on alfalfa gradually decreased, and all of them were in the first grade (good growth, not affected) on the 55th day. The death rates on the 62nd and 77th days were only 1.67% and 1.75%, respectively (Fig. 8A). After low-dose herbicide was sprayed at the seedling stage, the mortality rate was the highest on the 28th and 47th days (19.62% and 18.75%, respectively), and then the mortality rate gradually decreased (Fig. 8B).

Moderate-dose treatment of alfalfa damage before emergence

After a moderate dose of herbicide was sprayed at the seedling stage, the death rate of alfalfa was highest on the 47th day (24.98%), followed by that on the 28th day (22.37%). Although the death rate gradually decreased over time, the decline was slow, and 8.07% of the alfalfa still died on the 77th day. On the 28th and 47th days, 4.12% and 6.36% of the alfalfa plants were not affected, respectively. On the 55th and 62nd days, some of the alfalfa plants returned to normal growth, and by the 77th day, 64.97% of the alfalfa plants had grown normally, and 19.15% of the alfalfa plants were classified as Grade II. It is inhibited but continues to grow (Fig. 8C, D).

Treatment of alfalfa damage with a normal dose before emergence

The results revealed that, on the 21st day, alfalfa was the most strongly affected by spraying a high dose of herbicide

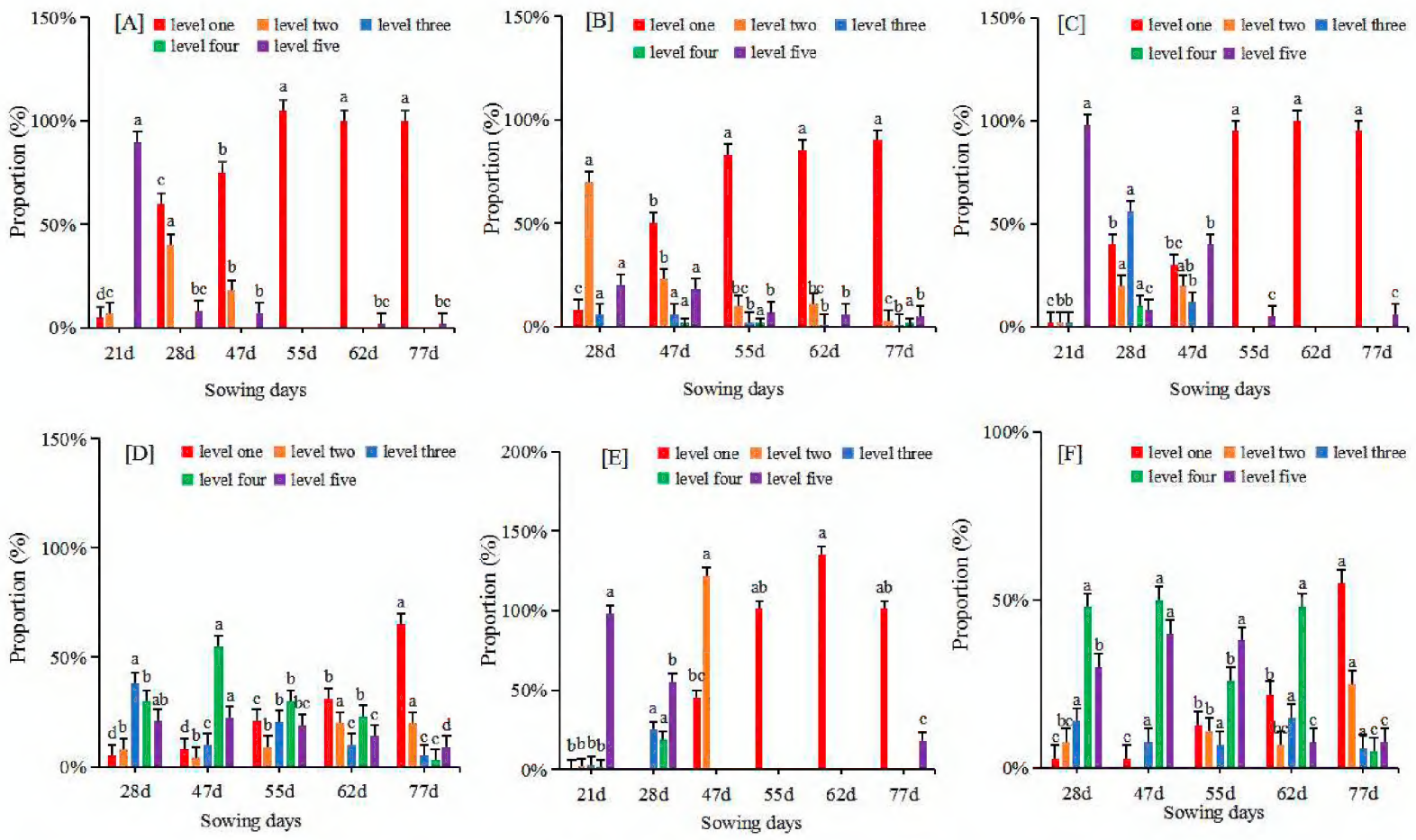

Figure 6. Changes with sowing date in terms of the degree of damage to low-dose, medium- and high-dose alfalfa. Note: The values indicate means (n = 3) with ± SE, and the same letters indicate nonsignificant differences at $p > 0.05$. Different letters indicate significant differences at $p < 0.05$.

before emergence: 97.07% of the alfalfa plants died completely, and only 0.53% of the alfalfa plants grew normally. Over time, the damage to the alfalfa gradually alleviated, and all the alfalfa plants returned to normal growth on the 55th day. Although there were deaths on day 77, the proportion of normally growing alfalfa was not significantly different from that on day 55 (Fig. 6E, Table 2). The mortality rate of alfalfa was 30.10% on the 28th day after spraying with a high dose of herbicide, reached its highest value on the 47th and 55th days (39.60% and 38.27%, respectively), and decreased to 8.01% and 7.96% on the 62nd and 77th days, respectively. The proportion of alfalfa that grew normally was the lowest on the 28th and 47th days (1.87% and 1.94%, respectively), and then the proportion gradually increased to 57.24% on the 77th day. At this time, 24.83% of alfalfa growth was still inhibited (Fig. 6F, Table 2).

Effects of spraying different doses of herbicides on the growth and yield of alfalfa at the seedling stage

Effects of spraying different doses of herbicides on the height of alfalfa plants at the seedling stage

The height of the alfalfa plants in the low-dose treatment group before 62 d increased to 81.23% at 77 d. However, the effects of the medium- and normal-dose treatments gradually increased with time but then decreased (Fig. 7A).

The results of the present study revealed that after three different doses of herbicide were sprayed at the first-year seedling stage, only the moderate dose and normal dose had significant effects on the height of alfalfa plants 45 days after seeding in the next year ($P < 0.05$) (Fig. 7B).

Effects of spraying different doses of herbicides on the stem diameter of alfalfa at the seedling stage

On day 28, the stem diameter of alfalfa treated with the three different doses decreased to 75.04%, 66.81% and 73.54% of that of the control. On day 47, the stem diameter of alfalfa treated with three different doses decreased by 75.78%, 61.04% and 59.22% compared with that of the control. On day 77, the stem diameter of the three treatment groups increased to 85.92%, 72.85% and 71.24% of that of the control group, respectively (Fig. 7C). The results revealed that spraying three different doses of herbicides in the first-year seedling stage had no significant effect on the stem diameter of alfalfa sown in the next year (Fig. 7D).

Effects of spraying different doses of herbicides at the seedling stage on the number of basal branches of alfalfa

After the plants were sprayed with different doses of herbicides at the seedling stage, the number of basal

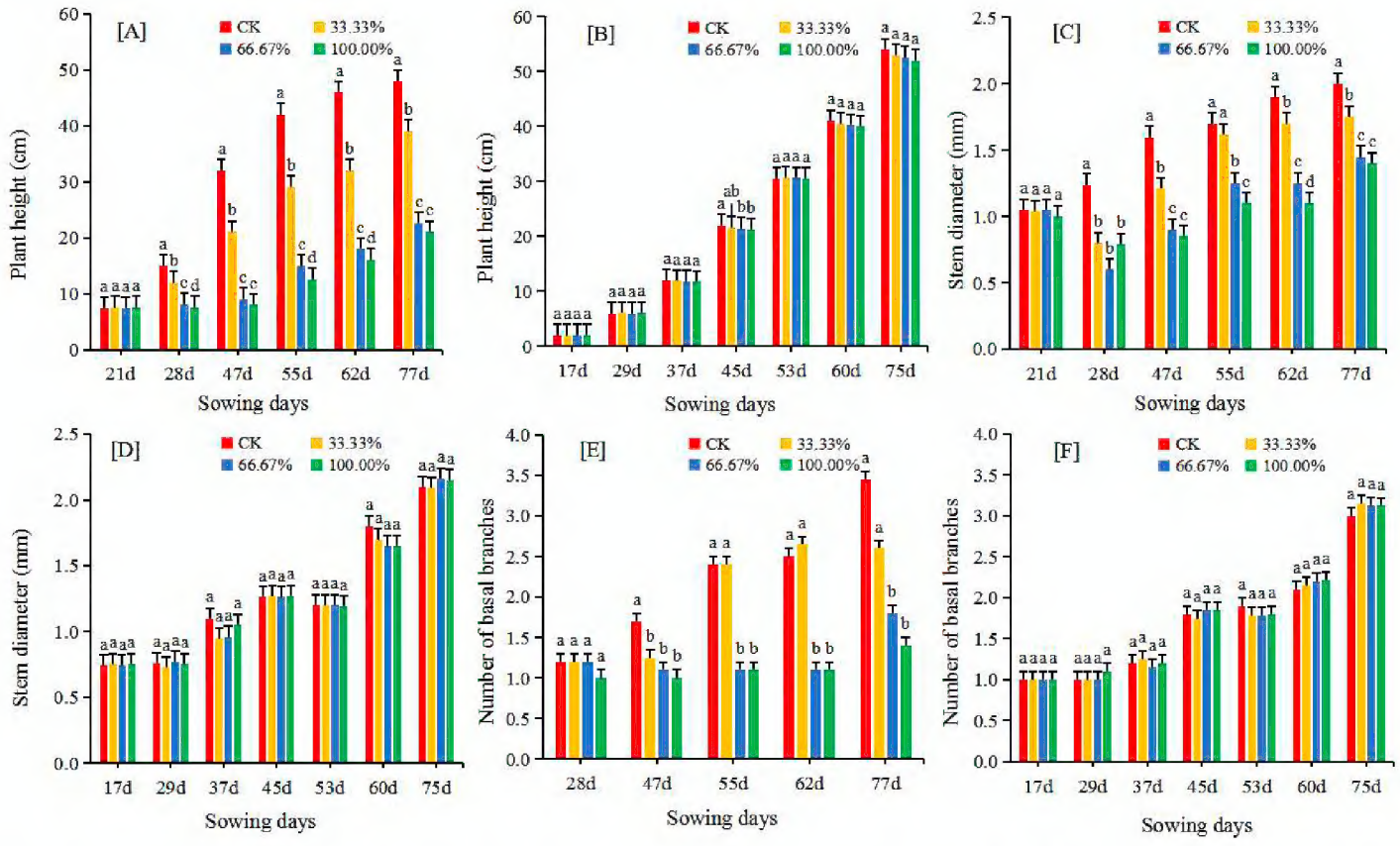

Figure 7. Effects of different spraying doses at the emergence stage on the height, diameter and basal branching of alfalfa plants. Note: The values indicate means (n = 3) with ± SE, and the same letters indicate nonsignificant differences at $p > 0.05$. Different letters indicate significant differences at $p < 0.05$.

Table 2. Effects of different spraying doses at preemergence on alfalfa yield and other indexes.

Spraying dose/%	Number of seedlings / Ten thousend plant·hm^{-2}	Aboveground fresh weight/kg·hm^{-2}	Aboveground dry weight/kg·hm^{-2}	Water content/%	Fresh weight per plant/g	Dry weight per plant/g
CK	269.68 ± 2.95^{a}	6227.99 ± 277.37^{a}	1717.59 ± 13.37^{a}	72.31 ± 1.24^{a}	2.31 ± 0.11^{c}	0.63 ± 0.01^{b}
33.33	37.04 ± 1.97^{b}	2182.48 ± 53.65^{b}	621.10 ± 41.39^{b}	71.58 ± 1.39^{a}	5.92 ± 0.33^{a}	1.68 ± 0.07^{a}
66.67	13.79 ± 0.66^{c}	730.22 ± 32.53^{c}	202.85 ± 8.96^{c}	72.22 ± 0.03^{a}	5.30 ± 0.08^{ab}	1.47 ± 0.02^{a}
100.00	8.91 ± 1.11^{c}	453.00 ± 54.51^{c}	130.12 ± 18.18^{c}	71.32 ± 1.32^{a}	5.11 ± 0.27^{b}	1.47 ± 0.14a

branches in the three alfalfa treatments significantly differed from that in the control ($P < 0.05$). The number of basal branches of alfalfa treated with the low dose was significantly different from that of the control on day 47 (69.39%) and 80.29% on day 77 (80.29%), but there was no significant difference between the two groups (Fig. 7E). The results revealed that spraying three different doses of herbicides in the first-year seedling stage had no significant effect on the number of basal branches of alfalfa sown in the next year (Fig. 7F).

Effects of spraying different doses of herbicides at the seedling stage on alfalfa yield and other indicators

On day 77, the number of plants per unit area of alfalfa in the control plot was 2,696,800 plants/hm2, and the number of plants per unit area in the low-dose, medium-dose and normal-dose treatments was 83.81%, 57.38% and 33.85% lower than that in the control plots, respectively. There were significant differences among the three doses. The above-ground fresh weight of alfalfa in the control plot was 6227.99 kg/hm^2, and the above-ground fresh weights in the three different treatment groups decreased to 70.36%, 32.08% and 11.25% of those in the control plot, respectively, and the differences among the three treatment groups were significant. The above-ground dry weight (yield) of the alfalfa in the control plot was 1717.59 kg/hm^2, the above-ground dry weights of the three different treatment groups decreased to 69.64%, 32.42% and 11.98% of those of the control plot, respectively, and the differences among the three different treatment groups were significant (Table 3). In the following year, the aboveground fresh weight of alfalfa in the control plot was 7101.49 kg/hm^2. The above-ground fresh weights of the alfalfa treated with the low, medium and normal doses were 90.87%, 90.04% and 95.17% greater than those of the control, respectively. In the following year, the ground dry weight of the alfalfa in the control plot was 2058.99 kg/hm^2, which was 91.87%, 87.07% and 93.20% lower than that of the control in the three different dose treatments, respectively (Table 4).

Effects of spraying different doses of herbicides at the branching stage on the growth and yield of alfalfa

Effects of spraying different doses of herbicides on the height of alfalfa plants at the branching stage

The height of the alfalfa plants seeded in the next year was significantly affected by the three different dose treatments in the first-year branching stage ($P < 0.05$), and the height of the alfalfa plants decreased with increasing dose. At 75 d, the height of the alfalfa plants in the control plot was 52.80 cm, and those in the three different treatment groups were 85.51%, 73.96% and 53.62% greater than those in the control group, respectively (Fig. 8A, B).

Effects of spraying different doses of herbicides on the stem diameter of alfalfa at the branching stage

The stem diameter of alfalfa in the 53 d control plots was 1.61 mm, which was 73.67%, 67.12% and 32.57% of that in the control plots, respectively, and increased to 89.03%, 80.35% and 73.69% of that in the control plots at 75 d (Fig. 8C, D).

Effects of spraying different doses of herbicides at the branching stage on the number of basal branches of alfalfa

The results showed that on day 77, the number of basal branches of alfalfa treated with the three different doses decreased by 84.41%, 70.59% and 74.41% compared with that of the control (Fig. 8E). The results revealed that at 75 d, the number of alfalfa basal branches in the control plot was 3.10, and those in the three treatments decreased by 79.03%, 72.58% and 54.84%, respectively, compared with those in the control (Fig. 8F).

Damage to alfalfa treated with a low dose at the branching stage

There was no significant change in the proportion of alfalfa that could grow normally (level 1 damage) or the proportion of alfalfa that died (level 5 damage) at 55, 62 and 77 days after low-dose herbicide was applied at the branching stage (Fig. 9A). After a low dose of herbicide was sprayed at the first-year branching stage, alfalfa sown in the next

Table 3. Effects of different spraying doses at the emergence stage on alfalfa yield and other indexes.

Spraying dose/%	Number of seedlings/ Ten thousand plant·hm^{-2}	Aboveground fresh weight/kg·hm^{-2}	Aboveground dry weight/kg·hm^{-2}	Water content/%	Fresh weight per plant/g	Dry weight per plant/g
CK	269.68 ± 2.95^a	6227.99 ± 277.37^a	1717.59 ± 13.37^a	72.31 ± 1.24^a	2.31 ± 0.11^a	0.63 ± 0.01^a
33.33	226.01 ± 2.95^b	4382.22 ± 94.16^b	1196.80 ± 17.18^b	72.67 ± 0.60^a	1.94 ± 0.03^b	0.53 ± 0.01^b
66.67	154.75 ± 8.70^c	1997.78 ± 61.86^c	556.82 ± 16.46^c	72.12 ± 0.52^a	1.30 ± 0.05^c	0.36 ± 0.02^c
100.00	91.29 ± 4.01^d	700.55 ± 20.13^d	205.82 ± 9.20^d	70.58 ± 1.48^a	0.77 ± 0.01^d	0.23 ± 0.01^d

Table 4. Effects of different spraying doses at the branching stage on alfalfa yield and other indexes.

Spraying dose/%	Number of seedlings/ Ten thousand plant·hm^{-2}	Aboveground fresh weight/kg·hm^{-2}	Aboveground dry weight/kg·hm^{-2}	Water content/%	Fresh weight per plant/g	Dry weight per plant/g
CK	269.68 ± 2.95^a	6227.99 ± 277.37^a	1717.59 ± 13.37^a	72.31 ± 1.24^a	2.31 ± 0.11^a	0.63 ± 0.01^a
33.33	240.48 ± 5.10^b	4088.55 ± 134.31^b	1143.43 ± 7.28^b	71.98 ± 0.77^a	1.70 ± 0.03^b	0.48 ± 0.01^b
66.67	200.40 ± 5.10^c	2488.11 ± 100.03^c	666.34 ± 30.04^c	73.23 ± 0.14^a	1.24 ± 0.07^c	0.33 ± 0.02^c
100.00	162.55 ± 4.01^d	1920.33 ± 74.93^d	521.56 ± 27.47^d	72.86 ± 0.59^a	1.18 ± 0.03^c	0.32 ± 0.01^c

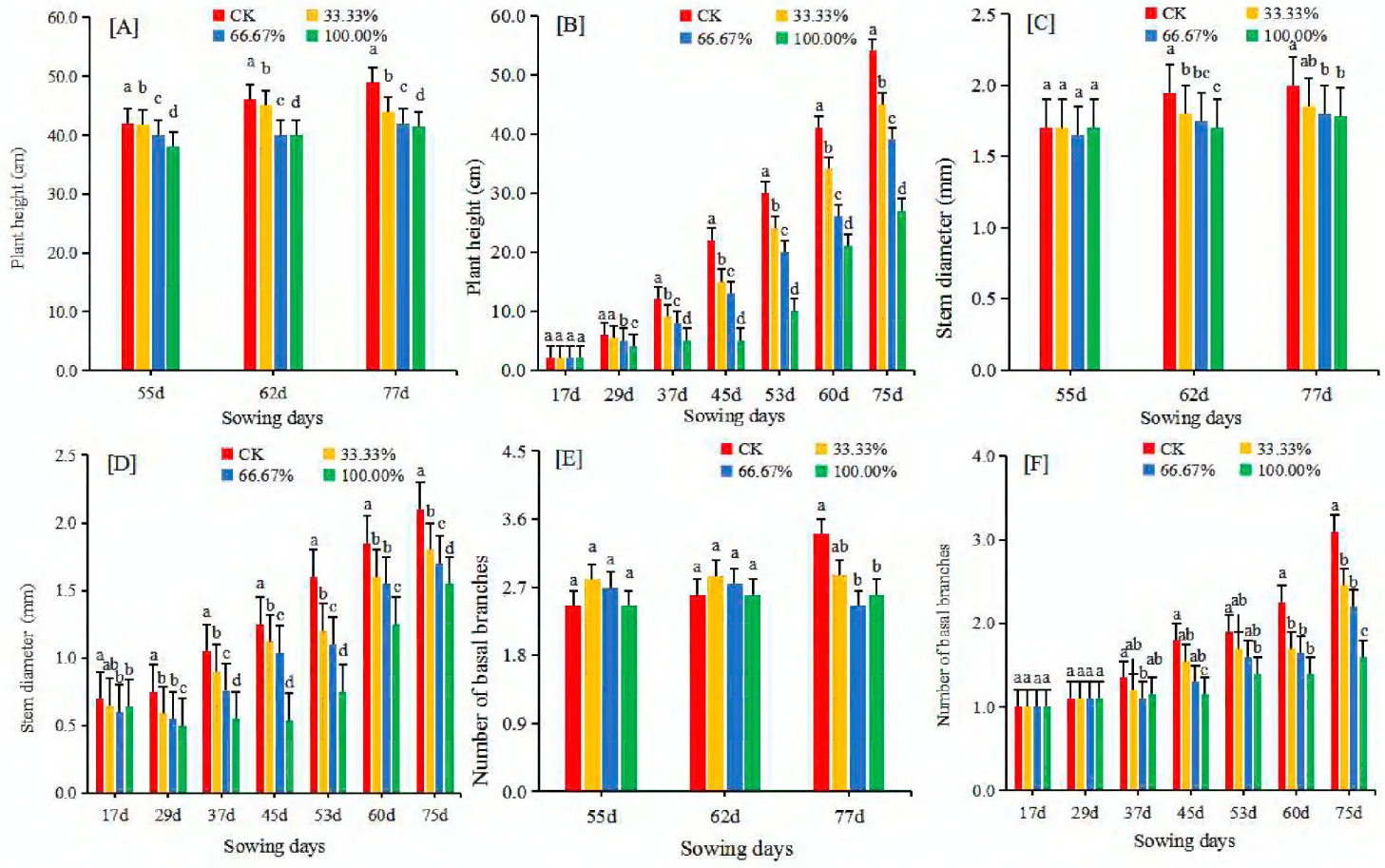

Figure 8. Effects of different spraying doses at the branching stage on plant height, stem diameter and number of basal branches of alfalfa Note: The values indicate means (n = 3) with ± SE, and the same letters indicate nonsignificant differences at $p > 0.05$. Different letters indicate significant differences at $p < 0.05$.

year was still harmful. The results revealed that the mortality rates at 17 d, 29 d and 37 d after sowing were the highest, at 8.86%, 10.42% and 10.07%, respectively (Fig. 9B).

Damage to alfalfa treated with a low dose at the branching stage

The proportion of secondary herbicides decreased gradually, from 74.09% at 55 d to 15.63% at 77 d, whereas the proportions of tertiary and tertiary levels increased gradually, from 7.05% and 1.16% at 55 d to 50.00% and 13.36% at 77 d, respectively. The harm caused by herbicides to alfalfa gradually increased (Fig. 9C). The results revealed that the mortality rates at 17 d, 29 d and 37 d after sowing were the highest, at 8.86%, 10.42% and 10.07%, respectively. The proportion of secondary, tertiary and tertiary alfalfa affected was the highest on the 29th day, while the proportion of normal growth (Grade I) alfalfa on the 29th day was the lowest (39.89%) (Fig. 9D).

Damage to alfalfa treated with a normal dose at the branching stage

After the plants were sprayed with a normal dose of herbicide at the branching stage, the percentage of alfalfa with Grade II damage gradually decreased from 62.64% on

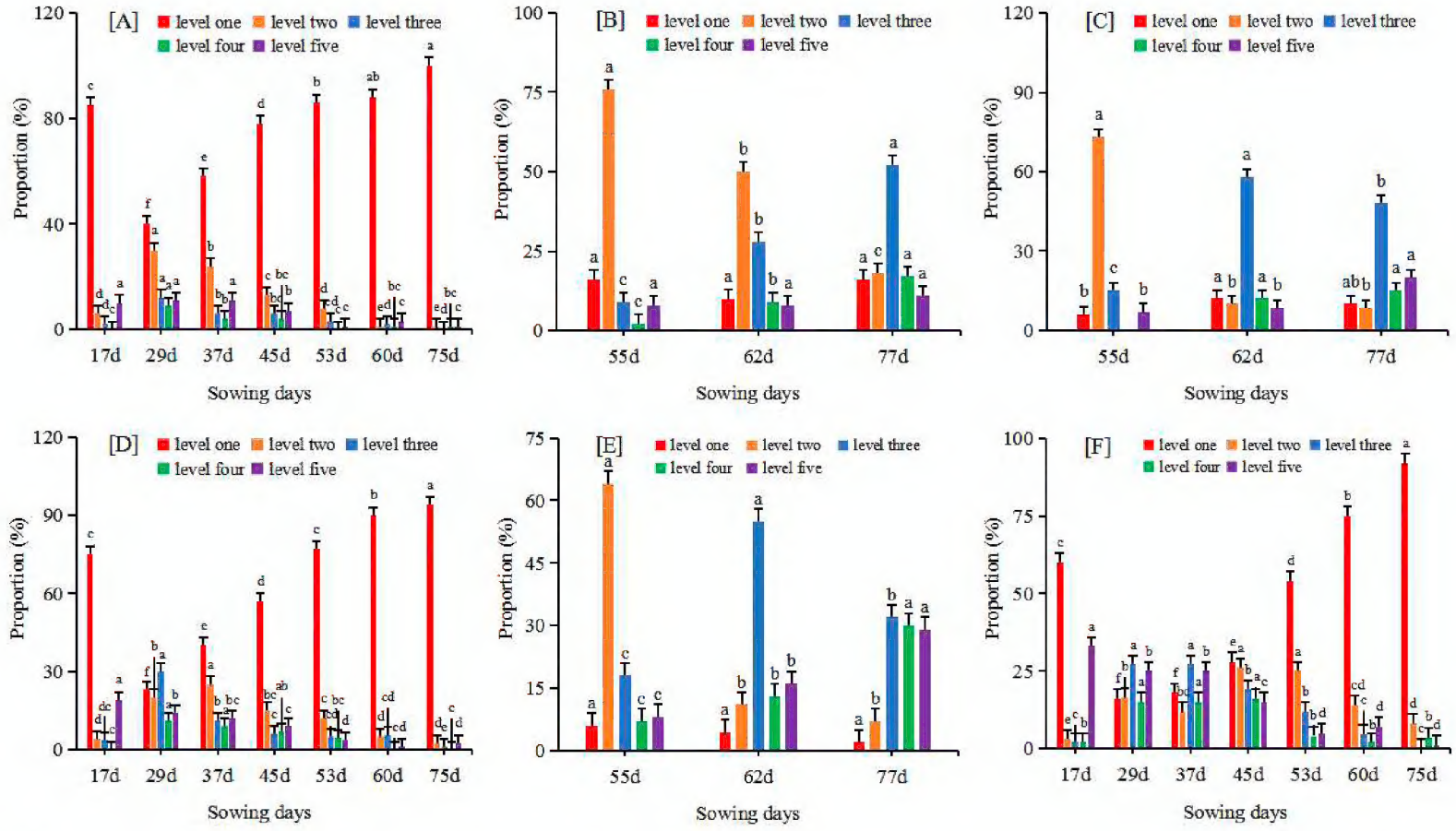

Figure 9. Changes with sowing date in terms of the degree of damage to low-dose, medium-dose and high-dose alfalfa. Note: The values indicate means (n = 3) with ± SE, and the same letters indicate nonsignificant differences at $p > 0.05$. Different letters indicate significant differences at $p < 0.05$.

day 55 to 6.80% on day 77, and the percentage of alfalfa with Grade III, 4 and 5 damage gradually increased from 18.50% on day 55. 5.56% and 8.13% increased to 32.51%, 30.57% and 29.14%, respectively, at 77 d, indicating a trend toward increasing herbicide damage (Fig. 9E). At 53 d, 24.63% of the alfalfa was classified as Grade II, and 14.67% was classified as Grade III. On day 75, 89.90% of the alfalfa plants grew normally (Fig. 9F).

Effects of spraying different doses of herbicides at the branching stage on alfalfa yield and other indexes

The number of plants per unit area in the low-dose, medium-dose and normal-dose treatments was lower than 89.17%, 74.31% and 60.28%, respectively, in the control plot. On day 77, the aboveground fresh weight of the alfalfa in the control plot was 6227.99 kg/hm^2, and the aboveground fresh weights of the alfalfa treated with the three different dosages decreased by 65.65%, 39.95% and 30.83%, respectively. On day 77, the aboveground biomass (yield) of alfalfa in the control plot was 1717.59 kg/hm^2, and the aboveground biomass of the plots treated with the three different dosages decreased by 66.57%, 38.80% and 30.37% of that of the control plot, respectively (Table 4). On day 75, the number of plants per unit area of alfalfa in the control plot was 2.04 million/hm^2, and the plots treated with three different dosages presented 83.59%, 60.13% and 36.66% fewer plants than did the control plots. The aboveground biomass (yield) of the alfalfa in the control plot was 2058.99 kg/hm^2, whereas that in the three plots treated with different doses decreased to 77.03%, 52.81% and 25.30% of that in the control plot, respectively; these values decreased significantly, and the difference was significant among the different treatments. The fresh weight per plant and biomass per plant were 88.98% and 71.11% of those of the control in the medium-dose and normal-dose treatments, respectively, which were significantly different from those of the control (Tables 5–7).

Discussion

Effects of different doses of herbicides on the germination of alfalfa seeds

Seed germination is the primary stage of seed plant establishment. In this study, we found that different herbicide treatments significantly decreased the germination of alfalfa. These findings align with those of previous studies reporting that the germination rate, germination potential and germination index of alfalfa gradually decrease with increasing concentrations of herbicides (Gaida and Brzęczek-Szafran 2020). Earlier, Bhattarai et al. (2021) reported that the germination rate and germination index of alfalfa decreased significantly with increasing herbicide application severity. On the other hand, the root–shoot ratio and vitality index of the alfalfa seedlings treated with the three different doses were significantly lower than those of the control seedlings. These findings indicate that herbicides significantly inhibited the growth of alfalfa seedlings, resulting in the inability of roots to extend and the ability to absorb water and nutrients, thus affecting their growth.

Proline, malondialdehyde (MDA) and soluble protein are important osmoregulatory substances in plant cells and play crucial roles in protecting cell membranes and maintaining cell life (Guo et al. 2023). Researchers have reported that under herbicide stress, the contents of proline and MDA first increase but then decrease with increasing herbicide concentration (Erdei 2022; Płonka et al. 2022). However, the results revealed that proline and MDA contents in alfalfa tended to decrease with increasing herbicide concentration. Low doses of herbicide may have exceeded the tolerance limit of alfalfa and destroyed the cell structure of alfalfa; thus, the accumulation of proline and MDA could not be observed. This study also revealed that different doses of herbicides reduced the soluble sugar content in alfalfa seedlings. This finding aligns with previous studies reporting that herbicides reduce the soluble sugar content (Wu et al. 2019). Spraying different doses of herbicide led to a significant decrease in soluble protein content in alfalfa, which aligns with earlier studies (Płonka et al. 2022). Many

Table 5. Effects of different spraying doses at preemergence on alfalfa yield and other indexes in the second year.

Spraying dose/%	Number of seedlings/ Ten thousand plant·hm^{-2}	Aboveground fresh weight/kg·hm^{-2}	Aboveground biomass/kg·hm^{-2}	Water content/%	Fresh weight per plant/g	Biomass per plant/g
CK	204.77 ± 3.33^{a}	7101.49 ± 155.09^{a}	2058.99 ± 70.67^{a}	71.01 ± 0.71^{a}	3.47 ± 0.10^{a}	1.01 ± 0.05^{a}
33.33	198.95 ± 4.48^{a}	6452.82 ± 143.08^{b}	1891.50 ± 67.79^{ab}	70.71 ± 0.84^{a}	3.25 ± 0.14^{a}	0.95 ± 0.05^{a}
66.67	192.95 ± 2.56^{a}	6393.94 ± 150.94^{b}	1792.82 ± 50.15^{b}	71.94 ± 0.78^{a}	3.32 ± 0.09^{a}	0.93 ± 0.03^{a}
100.00	203.45 ± 4.31^{a}	6758.14 ± 54.51a^{b}	1918.89 ± 35.26^{ab}	71.19 ± 0.90^{a}	3.33 ± 0.06^{a}	0.95 ± 0.03^{a}

Table 6. Effects of different spraying doses at the seedling stage on alfalfa yield and other indexes in the second year.

Spraying dose/%	Number of seedlings/ Ten thousand plant·hm^{-2}	Aboveground fresh weight/kg·hm^{-2}	Aboveground biomass/kg·hm^{-2}	Water content/%	Fresh weight per plant/g	Biomass per plant/g
CK	204.77 ± 3.33^{a}	7101.49 ± 155.09^{a}	2058.99 ± 70.67^{a}	71.01 ± 0.71^{a}	3.47 ± 0.10^{a}	1.01 ± 0.05^{a}
33.33	171.17 ± 2.74^{b}	5512.98 ± 222.75^{b}	1586.11 ± 55.67^{b}	71.58 ± 0.75^{a}	3.22 ± 0.10^{ab}	0.93 ± 0.02^{ab}
66.67	123.12 ± 4.42^{c}	3796.56 ± 116.39^{c}	1087.27 ± 25.62^{c}	71.72 ± 0.64^{a}	3.09 ± 0.06^{b}	0.89 ± 0.02^{b}
100.00	75.08 ± 3.68^{d}	1851.64 ± 102.26^{d}	520.87 ± 38.77^{d}	71.94 ± 0.65^{a}	2.47 ± 0.07^{c}	0.69 ± 0.04^{c}

Table 7. Effects of different spraying doses at the branching stage on alfalfa yield and other indexes in the second year.

Spraying dose/%	Number of seedlings/ Ten thousend plant·hm^{-2}	Aboveground fresh weight/kg·hm^{-2}	Aboveground biomass/kg·hm^{-2}	Water content/%	Fresh weight per plant/g	Biomass per plant/g
CK	204.77 ± 3.33^{a}	7101.49 ± 155.09^{b}	2058.99 ± 70.67^{b}	71.01 ± 0.71a	3.47 ± 0.10^{b}	1.01 ± 0.05^{b}
33.33	215.22 ± 6.56^{a}	9149.01 ± 247.49^{a}	2667.30 ± 81.99^{a}	70.85 ± 0.24a	4.25 ± 0.02^{a}	1.24 ± 0.01^{a}
66.67	217.72 ± 4.67^{a}	8921.96 ± 142.04^{a}	2656.73 ± 106.38^{a}	70.25 ± 0.81a	4.10 ± 0.05^{a}	1.22 ± 0.04^{a}
100.00	218.47 ± 2.25^{a}	9397.84 ± 149.55^{a}	2632.25 ± 32.34^{a}	72.22 ± 0.54a	4.31 ± 0.10^{a}	1.21 ± 0.02^{a}

studies have shown that the antioxidant enzyme system in plants plays a crucial role in maintaining plant stress resistance (Bowler et al. 1992; Stephenie et al. 2020; Rajput et al. 2021). We found that antioxidant activity increased in response to herbicide application, which is in line with earlier studies reporting the same findings (Maqbool et al. 2020).

Effects of spraying different doses of herbicides for different periods on the emergence and growth of alfalfa

Seed emergence time and relative emergence rate are important criteria for measuring seed emergence. A low relative emergence rate indicates that seeds do not germinate smoothly or die before emergence. The results revealed that spraying different doses of herbicide significantly reduced the emergence rate of alfalfa. This could be linked to herbicide-mediated increases in membrane damage and enzyme inactivation caused by increased ROS production. In the process of plant growth, plant height, stem diameter, and the number of basal branches are important indicators of plant growth (Zhang et al. 2024). The results of this study also revealed that spraying different doses of this herbicide before emergence, emergence and branching significantly reduced these traits. The decrease in yield traits was linked with a decrease in photosynthetic efficiency, root growth, nutrient and water content and less synthesis of osmolytes (Zhang et al. 2024). In the second year, there were almost no weeds in the field, the degree of competition pressure between alfalfa species was low, and alfalfa was able to obtain sufficient light and water, so its growth was greater. However, after three doses of herbicide were sprayed at the first-year branching stage, the plant height, stem diameter and number of basal branches of alfalfa sown in the next year were significantly lower than those of the control. This was associated with the late spraying time of herbicides in the first year, the close interval between the sowing time of the next year, and the presence of large amounts of herbicides in the soil.

Effects of spraying different doses of herbicides for different periods on alfalfa yield

Compared with those in the control plot, the relative seedling emergence rates of alfalfa in the first three treatments were lower, the plant density was lower, and the intraspecific competition pressure was lower. Thus, alfalfa can obtain sufficient light, water and other resources, which results in better yields. Moreover, the height of the alfalfa plants planted in the following year also increased. Although the three treatments in the first-year seedling stage had no significant effect on the number of alfalfa plants sown in the next year, water content or biomass per plant, the yield of alfalfa treated with the three different doses still decreased slightly or significantly compared with that of the control. This may be because a small amount of herbicide still remained in the soil, which did not significantly differ from the control but still inhibited the growth of alfalfa. Some studies have shown that spraying different doses of the same herbicide has different effects on crops (Saha 2019; Wu et al. 2019; Erdei 2022). In general, the degree of herbicide damage to crops tends to increase with increasing spraying dose. The results revealed that spraying a high dose of herbicide at the preemergence, emergence and branching stages resulted in increased mortality.

Although the degree of injury caused by the herbicide gradually weakened over time, the duration of injury caused by the high dose of herbicide was still longer than that caused by the other two doses, which therefore impacted the alfalfa. These findings are the same as earlier results showing that different concentrations of herbicides have harmful effects on alfalfa (Hijano et al. 2013). In addition, the same dose of herbicide sprayed on alfalfa during different growth periods had different effects on alfalfa. Regardless of low-, medium- or high-dose treatment, the death rate of alfalfa was the highest before emergence, and the death rate was lower at the emergence and branching stages. These results indicated that with increasing growth, the tolerance of alfalfa to this herbicide gradually increased due to increased antioxidant activity. In the first-year branch period (August 2018), the plots treated with different herbicide doses experienced obvious herbicide injury to alfalfa in the following year, and the influence of herbicide injury tended to increase with increasing herbicide dose in the first year. This result may be due to the short interval between the application of herbicides and the sowing time of the next year and the herbicide residues in the soil. The low rainfall during the interval also resulted in only a small number of herbicides being washed away by rain, while a large amount of herbicides remained in the soil.

Conclusions

Compared with those in the control treatment, the germination rate, germination potential and germination index of alfalfa in the moderate- and high-dose treatments significantly decreased. The root-to-shoot ratio, activity index, soluble sugar content and soluble protein content of

the alfalfa in the three treatment groups significantly decreased, whereas the contents of three antioxidant enzymes significantly increased. In conclusion, although early alfalfa seedlings can clear reactive oxygen species to cope with stress by increasing the activities of POD, SOD and CAT enzymes, the contents of soluble sugars and soluble proteins in these plants are also significantly reduced, resulting in the abnormal growth of alfalfa seedlings. Compared with the control, spraying corn herbicide at the preemergence, emergence and branching stages caused herbicide injury to alfalfa, resulting in a significant reduction in the emergence rate of alfalfa, an increase in plant height and stem diameter, a decrease in biomass per plant, and ultimately a significant reduction in the biomass of alfalfa. However, with the growth of alfalfa, its tolerance to this herbicide improved. The mortality rate of alfalfa in the first-year branching period was the lowest, and the effects on plant height, stem diameter and yield were the lowest; however, the mortality rate was still significantly lower than that of the control. The effects of spraying different doses of this corn herbicide on alfalfa during the three periods of the first year tended to increase with increasing application dose.

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Conflicts of interest

The authors declare no conflicts of interest.

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